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Differential non-target-derived repulsive signals play a critical role in shaping initial axonal growth of dorsal root ganglion neurons

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Abstract

Initial trajectories of dorsal root ganglion (DRG) axons are shaped by chemorepulsive signals from surrounding tissues. Although we have previously shown that axonin-1/SC2 expression on DRG axons is required to mediate a notochord-derived chemorepulsive signal (T. Masuda et al., 2000, *Dev. Biol.* 224, 112–121), other molecules involved in the non-target-derived repulsive signals are largely unknown. Using coculture assays composed of tissues derived from the chick embryo or mutant mice treated with function-blocking antibodies and phosphatidylinositol-specific phospholipase C, we report here that the chemorepellent semaphorin 3A (Sema3A) and its receptor neuropilin-1 are required for mediating the dermamyotome- and notochord-derived, but not the ventral spinal cord-derived, chemorepulsive signal for DRG axons. The dermamyotome-derived chemorepulsion is exclusively dependent on Sema3A/neuropilin-1, whereas other molecules are also involved in the notochord-derived chemorepulsion. Chemorepulsion from the ventral spinal cord does not depend on Sema3A/neuropilin-1 but requires axonin-1/SC2 to repel DRG axons. Thus, differential chemorepulsive signals help shape the initial trajectories of DRG axons and are critical for the proper wiring of the nervous system.
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Introduction

The establishment of neuronal circuits depends critically on the axonal projection to their proper targets. During development, growth cones of extending axons are guided to their targets by four main axonal guidance mechanisms: contact attraction, contact repulsion, chemoattraction, and chemorepulsion (reviewed in Tessier-Lavigne and Goodman, 1996). The axonal projection of dorsal root ganglion (DRG) neurons is a useful model to examine the role of axonal guidance mechanisms. During the initial stage of DRG axonal growth, surrounding "nontarget" tissues such as the dermamyotome, the notochord, and the ventral spinal cord release strong chemorepulsive signals for DRG axons (Keynes et al., 1997; Nakamoto and Shiga, 1998). However, chemorepellents and their receptors that are responsible for these repulsive events are not fully understood. One of the best-characterized chemorepellents is semaphorin 3A (Sema3A/SEMA3A; hereafter referred to as Sema3A), a

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member of the semaphorin family that acts via the neuropilin-1 receptor (see Fujisawa and Kitsukawa, 1998; Kolodkin, 1998; Raper, 2000 for reviews). In *Sema3A* and *neuropilin-1* null mutants, aberrant axonal projections occur in neurons from the lateral part of DRGs projecting toward the dermamyotome, suggesting that Sema3A/neuropilin-1 plays an important role in the dermamyotome-derived chemorepulsion (Kitsukawa et al., 1997; Taniguchi et al., 1997; White and Behar, 2000). Because Sema3A is expressed in the dermamyotome and neuropilin-1 in DRG axons, these studies suggest that the dermamyotome-derived chemorepulsion for DRG axons is dependent on the Sema3A/neuropilin-1 signaling. However, the molecules responsible for chemorepulsion from the notochord and the ventral spinal cord are less clear; axonal projections from the DRG in these regions appear normal in *Sema3A* and *neuropilin-1* mutants (Kitsukawa et al., 1997; Taniguchi et al., 1997; White and Behar, 2000).

Recently, we have demonstrated that axonin-1/SC2, a member of the immunoglobulin superfamily of cell adhesion molecules, is expressed on growing DRG axons and mediates notochord-derived chemorepulsion (Masuda et al., 2000). In cocultures of DRGs and notochords, an antibody against axonin-1/SC2 diminished the response of DRG axons to notochord-derived chemorepulsion. However, the axonin-1/SC2 antibody failed to completely block the notochord-derived chemorepulsion, suggesting that other repulsive signals may also be involved (Masuda et al., 2000). The role of axonin-1/SC2 in chemorepulsion from the ventral spinal cord and the dermamyotome is unknown. In the present study, we have examined the role of Sema3A, neuropilin-1, and axonin-1/SC2 using collagen gel cocultures in which the function of these molecules has been perturbed. We found the differential contributions of Sema3A and neuropilin-1 to chemorepulsion from the dermamyotome, the notochord, and the ventral spinal cord. Furthermore, we also found that axonin-1/SC2 is involved in the ventral spinal cord-derived chemorepulsion. These findings indicate that both Sema3A/neuropilin-1-dependent and -independent mechanisms play a critical role in the formation of the initial trajectories of DRG axons.

Materials and methods

Animals

Fertilized chicken eggs were obtained from a local farm and incubated at 37.6°C, until they reached the appropriate ages [embryonic day 2 (E2) to E5]. The generation and the identification of *neuropilin-1*-deficient and *Sema3A*-deficient mice were performed as described (Kitsukawa et al., 1997; Taniguchi et al., 1997). E0.5 was defined midday of the day of vaginal plug discovery.

Expression of Sema3A in COS cells

COS-7 cells were grown to 80% confluence in a 35-mm dish (Sumitomo Bakelite, Tokyo, Japan) in Dulbecco's modified Eagle's medium (DMEM; Celox Laboratories, St. Paul, MN) with 10% calf serum (Invitrogen Corp., Carlsbad, CA) and transfected with pAG3-*myc-his-Sema3A* (a gift from Dr. J.A. Raper, the University of Pennsylvania School of Medicine) using Effectene transfection reagent (Qiagen, Hilden, Germany). Approximately 0.8 μg of DNA and 8 μl of Effectene reagent were added per 35-mm dish and the cells expressing Myc-His-tagged Sema3A were allowed to grow for 1 day. COS cell aggregates were prepared by the hanging drop method as previously described (Kennedy et al., 1994). The expression of Sema3A was confirmed by Western blot using an anti-His_6 antibody (Roche Diagnostics GmbH, Mannheim, Germany).

Collagen gel cultures

Notochords, dermamyotomes, and ventral spinal cords were dissected from E3 (stage 18–19 of Hamburger and Hamilton, 1951) chick embryos and E11.5 mouse embryos. DRG explants from thoracic segments were dissected out from E4 (stage 23–24) or E5 (stage 26–27) chick embryos and E13.5 mouse embryos. Pioneer DRG axons begin to extend, avoiding the notochord, the dermamyotome, and the ventral spinal cord, at around E3 and E10.5 in the chick and the mouse embryo, respectively (Ozaki and Snider, 1997; Shiga et al., 2000). We used E4/5 chick and E13.5 mouse DRG explants in this study, because it was difficult to isolate younger DRG explants from the surrounding mesenchymal tissues, and younger DRG explants were not suitable for quantitative analyses because of their more fasciculated axon bundles. DRG explants were embedded in a collagen gel approximately 200 or 300 μm distant from the explants and the aggregates of mock-transfected COS cells or COS cells transfected with pAG3-*myc-his-Sema3A*. In the case of combinations of mouse notochord explants with chick DRG explants, we cocultured three notochord explants with a single DRG explant, because the murine notochord-derived chemorepulsion is weaker than that of the chick in a collagen gel. Cultures were incubated at 37°C for 24–36 h in DMEM containing 10% heat-inactivated fetal calf serum (Invitrogen Corp.), 50 ng/ml 7S nerve growth factor (NGF; Chemicon, Temecula, CA), and 50 ng/ml neurotrophin-3 (NT-3; Sigma, St. Louis, MO). In some cases, cocultures were incubated for more than 48 h to examine whether DRG axons can reach the explants. For function-blocking studies, polyclonal antibodies against neuropilin-1 (1:40; He and Tessier-Lavigne, 1997) and axonin-1/SC2 (a gift from Dr. M. Grumet, the State University of New Jersey) (10 μg/ml; Lustig et al., 1999; Masuda et al., 2000; Shiga et al., 1997) were added to the culture medium. To remove glycosylphosphatidylinositol (GPI)-anchored membrane proteins including axonin-1/SC2 from DRG ax-

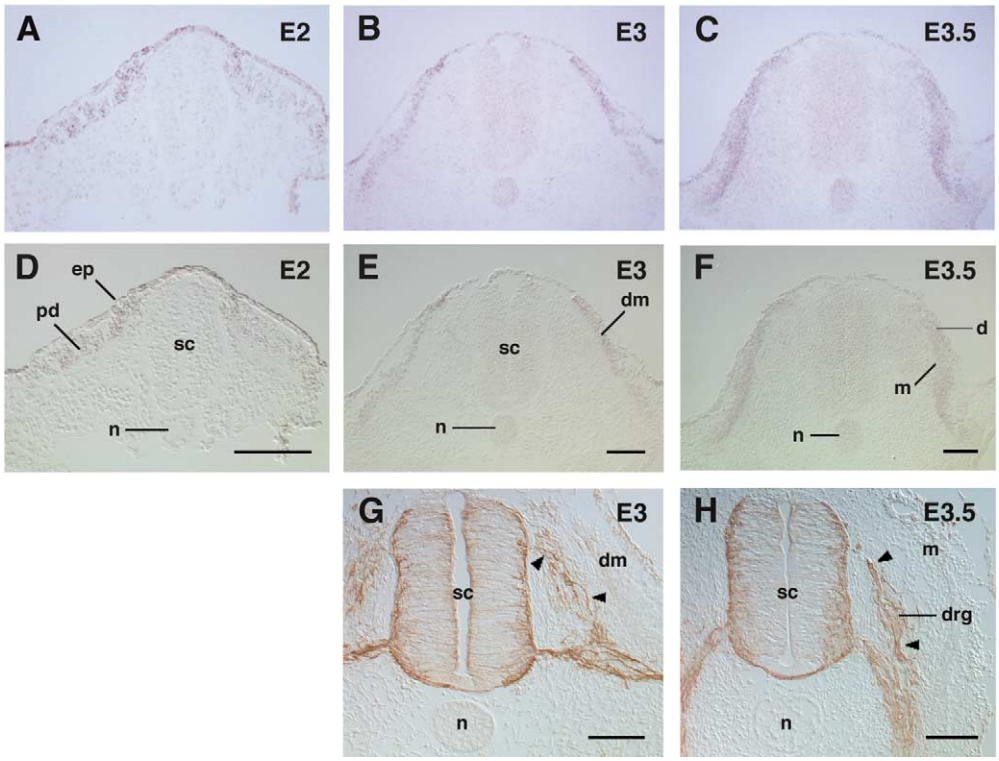

Fig. 1. The distribution of *Sema3A* mRNA in thoracic segments at early stages of the chick embryo (A–C) and corresponding Nomarski images (D–F). (A, D) At stage 14 (E2), *Sema3A* expression was detected in the presumptive dermamyotome (pd) and the epidermis (ep). (B, E) At stage 18 (E3), the dermamyotome (dm) and the epidermis continued to express *Sema3A* signals at a high level. Weak *Sema3A* expression began to be detected in the notochord (n). The spinal cord (sc) showed no detectable signal for *Sema3A.* (C, F) At stage 21 (E3.5), *Sema3A* expression remained detectable in the dermatome (d), the myotome (m), and the epidermis. *Sema3A* signals increased in the notochord, and the spinal cord began to express *Sema3A* weakly. (G, H) β-tubulin-stained axons (arrowheads) of DRGs (drg) at stage 18 (E3; G) and at stage 21 (E3.5; H). Scale bars: 100 μm.

onal membranes, 50 mU/ml phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis* (Funakoshi Co., Tokyo, Japan) was added to the culture medium three times at intervals of 8–10 h during the culture period (Masuda et al., 2000).

Immunohistochemistry in vitro

Cultures were fixed for several days with 4% paraformaldehyde (PFA) in 0.1 M PB. Collagen gels containing tissue explants were then excised and processed for whole-mount immunohistochemistry (Masuda et al., 2000). After incubation with a blocking solution containing 2% normal horse serum (Invitrogen Corp.) and 0.1% Triton X-100 in PB, tissue explants were incubated with an anti-β-tubulin antibody (1 μg/ml; Promega Corp., Madison, WI) overnight, followed by a biotinylated secondary anti-mouse IgG antibody (Vector Laboratories, Burlingame, CA) for 4 h and a peroxidase-conjugated avidin–biotin complex (Vector Laboratories) for 2 h. They were then reacted with diaminobenzidine (DAB) using the ImmunoPure metal enhanced DAB substrate kit (Pierce, Rockford, IL). In some cases, anti-Islet-1 antibody (40.2D6, Developmental Studies Hybridoma Bank) was used as the primary antibody to label cells in the ventral spinal cord explants. All incubations were carried out at room temperature.

Analysis of DRG axonal growth

After staining DRG axons, the length and the trajectory of the axons were traced using a camera lucida (Nikon, Tokyo, Japan). Axons from DRG explants cocultured with surrounding tissues or COS cell aggregates were grouped into four quadrants: proximal, distal, and two lateral quadrants (see Figs. 2–6). The lengths of the five longest axons were measured in the proximal and distal quadrants for each culture (Lumsden and Davies, 1983). Data were statistically analyzed using Student's unpaired *t* test and ANOVA.

In situ hybridization

Digoxigenin (DIG)-labeled RNA probes were synthesized in vitro against the full-length chick *Sema3A* cDNA (Shepherd et al., 1996), using the DIG RNA labeling kit (Roche Diagnostics GmbH) as directed. Thoracic segments of E2–4 (stage 14–24) chick embryos were dissected out and frozen directly in Tissue-Tek OCT compound (Miles Inc., Elkhart, IN). Transverse sections (10-μm thickness)

were cut using a cryostat and collected onto 2% silane-coated slides. After fixation with 4% PFA in PBS for 10 min, sections were digested with proteinase K (10 μg/ml; Sigma) for 5 min and refixed with 4% PFA in PBS for 5 min. Acetylation was carried out for the next 10 min by treatment with 0.1 M triethanolamine (Wako Pure Chemical Industries, Osaka, Japan) and acetic anhydride (Wako). After dehydration in a series of increasing ethanol concentrations, sections were hybridized for 20 h at 55°C with DIG-labeled antisense or sense RNA probes (600–700 ng/ml) in a hybridization buffer containing 50% formamide (Wako) and 10% dextran sulfate (Sigma). Prior to immunohistochemical detection, sections were washed in 2× SSC at 37°C for 30 min, and in 50% formamide in 2× SSC at 55°C for 30 min. After treatment with RNase A (50 μg/ml; Roche Diagnostics GmbH) in RNase buffer (1 mM EDTA, 10 mM Tris–HCl, 500 mM NaCl) at 37°C for 30 min, sections were incubated in a blocking solution [1% blocking reagent (Roche Diagnostics GmbH) and 0.05% Triton X-100 in 2× SSC] for 2–3 h at room temperature. Sections were then incubated overnight at 4°C with alkaline phosphatase-conjugated anti-DIG Fab fragments (1:1000; Roche Diagnostics GmbH) and reacted with a solution containing 4-nitro blue tetrazolium chloride (NBT; Roche Diagnostics GmbH) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Roche Diagnostics GmbH) at room temperature and in the dark.

Immunohistochemistry in vivo

E3 (stage 18) or E3.5 (stage 21) chick and E11.5 mouse thoracic segments were fixed with 4% PFA overnight. After immersion in graded sucrose solutions in PB, tissues were frozen in Tissue-Tek OCT compound, and 14-μm transverse sections were cut using a cryostat and collected onto gelatin-coated slides. Cryostat sections were incubated for 1 h with a blocking solution containing 2% normal goat or horse serum (Invitrogen Corp.) and 0.1% Triton X-100 in PB. After incubation with anti-neuropilin-1 antibody (2 μg/ml; Takagi et al., 1995) or anti-β-tubulin antibody (500 ng/ml; Promega Corp.) overnight at 4°C, they were incubated for 1 h with a biotinylated secondary anti-rabbit or anti-mouse IgG antibody (Vector Laboratories), followed by the peroxidase-conjugated avidin–biotin complex (Vector Laboratories) for 1 h. The sections were then reacted with DAB using the ImmunoPure metal enhanced DAB substrate kit. All incubations were carried out at room temperature except for the primary antibody.

Results

Expression of Sema3A mRNA in the tissues surrounding DRGs

Pioneer DRG axons begin to extend bidirectionally toward their central and peripheral targets at around E3 (stage 18–20) in the chick and E10.5 in the mouse (Ozaki and Snider, 1997; Shiga et al., 2000). To begin to study the role of Sema3A, we first examined the distribution of *Sema3A* mRNA in the chick embryos using a DIG-labeled antisense RNA probe. Although *Sema3A* expression has been examined in the early developing chick and mouse embryos (Adams et al., 1996; Püschel et al., 1996; Shepherd et al., 1996), we specifically examined expression in tissues surrounding the DRG during the initial growth of chick DRG axons.

At E2 (stage 14), before DRGs are formed, *Sema3A* expression was detected in the presumptive dermamyotome and the epidermis in thoracic segments (Fig. 1A and D), consistent with a previous study (Shepherd et al., 1996). At this stage of development, no signal was detected within the notochord or spinal cord. At E3 (stage 18) when pioneer DRG axons extend bidirectionally (Fig. 1G), *Sema3A* expression was detected in the dermamyotome and the epidermis and now the notochord began to express *Sema3A*, although more weakly than in the dermamyotome (Fig. 1B and E). Virtually no expression was observed within the spinal cord. At E3.5 (stage 21), DRG axons extended further both centrally and peripherally (Fig. 1H). The dermamyotome is differentiated into the superficial dermatome and the deep myotome, both of which expressed *Sema3A*. The *Sema3A* signal became more intense in the notochord and weak expression was now seen throughout the spinal cord (Fig. 1C and F). After E4 (stage 24), the *Sema3A* signal in the spinal cord became localized to the ventral region (data not shown), in accordance with previous reports (Messersmith et al., 1995; Shepherd et al., 1996, 1997; Wright et al.,

Table 1
Axon outgrowth from DRGs cocultured with COS cell aggregates secreting Sema3A

Coculture conditions	Mean outgrowth ± SEM (μm)		Mean *p/d* value ± SEM	*N*	*P* value (Student's *t* test)
	Proximal quadrant (*p*)	Distal quadrant (*d*)			
E4 DRG + COS-Sema3A	17.7 ± 6.4	143 ± 9.3	0.124 ± 0.04	18	<0.0001
E4 DRG + COS-Mock	236 ± 18	252 ± 30	0.975 ± 0.21	11	NS (>0.05)
E5 DRG + COS-Sema3A	58.6 ± 7.9	328 ± 22	0.181 ± 0.02	28	<0.0001
E5 DRG + COS-Mock	327 ± 10	354 ± 9.5	0.941 ± 0.04	31	NS (>0.05)

Note. NS, not significant.

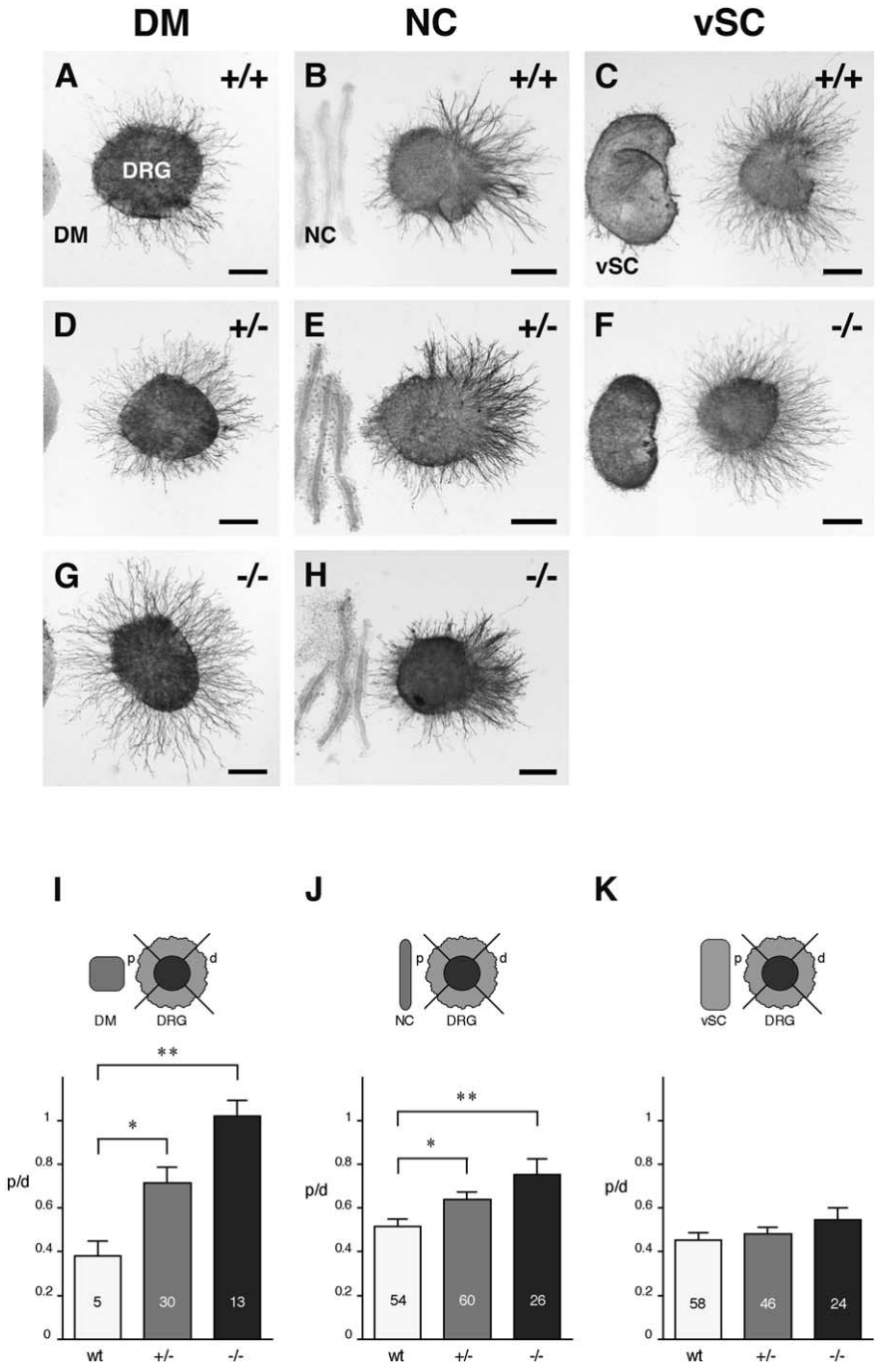

Fig. 2. *Sema3A*-deficient dermamyotomes and notochords lose or reduce their repulsive activities for DRG axons. (A, D, G) Dermamyotomes (DM) from *Sema3A*+/− (D) or *Sema3A*−/− (G) mice reduced their chemorepulsive activities in a Sema3A-dose-dependent manner, compared to those of *Sema3A*+/+ dermamyotomes (A). (B, E, H) Chemorepulsive activities derived from *Sema3A*+/− (E) or *Sema3A*−/− (H) notochords were partially reduced in a dose-dependent manner compared to those from *Sema3A*+/+ notochords (NC; B). (C, F) Ventral spinal cords (vSC) from *Sema3A*−/− mice (F) showed no significant reduction in their chemorepulsive activities for DRG axons compared to those of the *Sema3A*+/+ ventral spinal cord (C). (I–K) The quantification of chemorepulsive activities for DRG axons. The length of E5 DRG axons growing toward the tissue explants (*p*) was compared to that away from them (*d*). The *p/d* values significantly increased in the groups cocultured with *Sema3A*+/− or *Sema3A*−/− dermamyotomes and notochords (I, J). No significant increase was observed in either group cocultured with *Sema3A*+/− and *Sema3A*−/− ventral spinal cords (K). The bars represent means + SEM and the number of cocultures is shown in the bar. $*P < 0.05$, $**P < 0.001$, compared to the control (wild-type) group. Scale bars: 200 μm.

1995). Control sections hybridized with a DIG-labeled sense probe and developed in parallel had no detectable signals (data not shown). These expression patterns suggest that Sema3A may be involved in both dermamyotome- and notochord-derived chemorepulsion during initial DRG axonal growth.

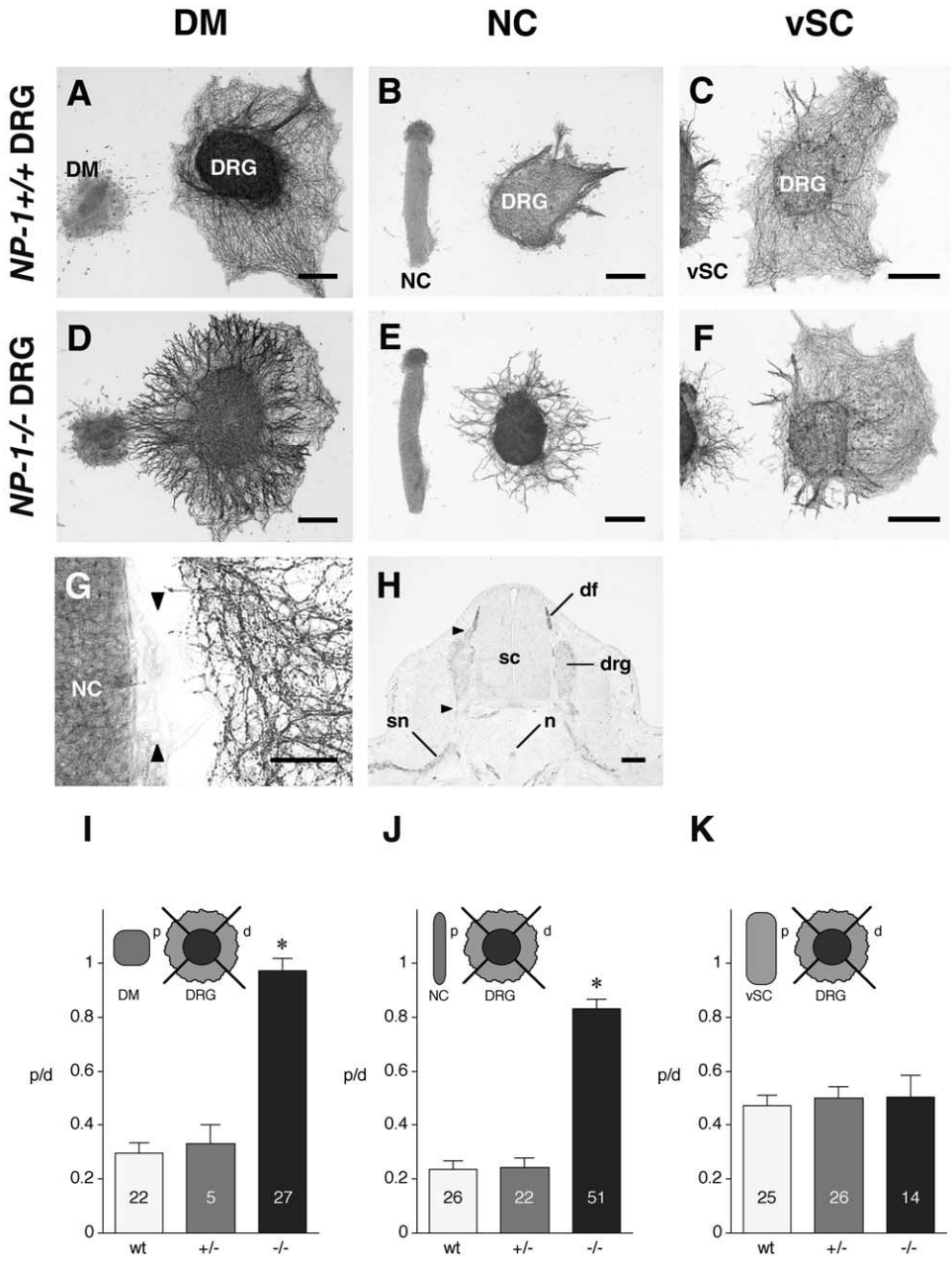

Fig. 3. DRG axons from *neuropilin-1*-deficient mice do not respond to dermamyotome- and notochord-derived repulsion. (A–G) E13.5 DRG explants from *neuropilin-1−/− (NP-1 −/−)* or *neuropilin-1+/+* (*NP-1 +/+*) mice were cocultured with E3 chick dermamyotome (DM), notochord (NC), or ventral spinal cord explants (vSC). (A–C) All these explants chemorepelled DRG axons from *neuropilin-1+/+* mice. (D) *Neuropilin-1−/−* DRG axons completely lost responsiveness to the dermamyotome-derived chemorepulsion. (E) *Neuropilin-1 −/−* DRG axons exhibit greatly reduced responsiveness to notochord-derived chemorepulsion. (F) *Neuropilin-1 −/−* DRG axons still retain the responsiveness to ventral spinal cord-derived chemorepulsion. (G) In the 48-h cocultures, *neuropilin-1−/−* DRG axons did not reach the notochord explants, leaving an axon-free zone. Arrowheads indicate the axon-free zone. (H) The expression of neuropilin-1 protein in thoracic segments of an E11.5 mouse embryo. Immunoreactivity was seen on DRGs (drg) and their axons (arrowheads). df, dorsal funiculus; n, notochord; sc, spinal cord; sn, spinal nerve. (I–K) Quantification of the chemorepulsive activities derived from the dermamyotome (I), the notochord (J), and the ventral spinal cord (K) for *neuropilin-1+/+*, *neuropilin-1+/−*, and *neuropilin-1 −/−* DRG axons. The bars represent means + SEM and the number of cocultures is shown in the bar. **P* < 0.0001, compared to the control (wild-type) group. Scale bars: 200 μm (A–F), 50 μm (G), 100 μm (H).

Loss or reduction of chemorepulsive activities for DRG axons from Sema3A- deficient dermamyotome and notochord

Based on the normal pattern of *Sema3A* expression, we next examined the contribution of Sema3A to the chemorepulsive signals derived from the dermamyotome, the notochord, and the ventral spinal cord. Because the chemorepulsive activity of Sema3A on early developing chick DRG axons has not been studied (Shepherd et al., 1996, 1997), we first examined whether Sema3A can repel DRG axons at these early stages. We cocultured E4 or E5 chick DRG

explants with aggregates of COS cells transfected with a Sema3A expression construct for 1 day in a collagen gel. For quantitative analysis, the DRG axonal length in the proximal quadrant (*p*), toward COS cell aggregates, was compared to that in the distal quadrant (*d*), away from the COS cells. The axonal outgrowth ratio *p/d* value is a measure of the repulsive activity, with ratios of 0 and 1 indicating complete and no repulsion, respectively. *Sema3A*-transfected COS cell aggregates significantly reduced the length of outgrowth in the proximal quadrant of both E4 and E5 DRG axons compared with that in the distal quadrant (Table 1, see also Fig. 6C). Mock-transfected COS cell aggregates had no apparent effect on E4/5 DRG axonal outgrowth (Table 1, see also Fig. 6E). Thus, these studies indicate that Sema3A repels DRG axons at early stages.

Next, we cocultured E5 chick DRG explants with dermamyotome, notochord, or ventral spinal cord explants from E11.5 *Sema3A*-deficient (*Sema3A*−/−) mice. The DRGs extended much shorter axons toward the wild-type (*Sema3A*+/+) dermamyotome, notochord, and ventral spinal cord, resulting in *p/d* values of 0.380, 0.518, and 0.453, respectively (Fig. 2A–C and 2I–K). By contrast, DRGs cocultured with the dermamyotome and the notochord from heterozygous *Sema3A* (*Sema3A*+/−) or *Sema3A*−/− mice had significantly increased *p/d* values of 0.715 (*Sema3A*+/− dermamyotome), 1.021 (*Sema3A*−/− dermamyotome), and 0.637 (*Sema3A*+/− notochord), 0.755 (*Sema3A*−/− notochord), indicating Sema3A-dose-dependent reductions in chemorepulsive signals (Fig. 2D, E, G, H, I, and J). Cocultures of DRGs with *Sema3A*+/− and *Sema3A*−/− ventral spinal cords showed no significant reduction in chemorepulsive actions [the *p/d* values were 0.453 (+/+), 0.481 (+/−), and 0.548 (−/−), $P = 0.129$ between the homozygous group and wild-type group] (Fig. 2F and K).

The *Sema3A*−/− dermamyotome showed a complete loss of chemorepulsive activity, consistent with the *in vivo* observation that aberrant projections occur in DRG axons projecting to the dermamyotome in *Sema3A*-deficient mice (Taniguchi et al., 1997; White and Behar, 2000). These results strongly suggest that dermamyotome-derived chemorepulsive activities are exclusively dependent on Sema3A. Although no abnormal projections toward the notochord were observed in vivo in *Sema3A*-deficient mice (Taniguchi et al., 1997; White and Behar, 2000), chemorepulsive activities derived from the *Sema3A*−/− notochord were partially reduced in our in vitro study. Taken together with the normal *Sema3A* mRNA expression pattern, these results suggest that the notochord secretes Sema3A as well as other chemorepellents. The ventral spinal cord at this early stage appears to secrete chemorepulsive molecules other than Sema3A, whereas at later stages the ventral spinal cord produces Sema3A which repels DRG axons (Messersmith et al., 1995; Püschel et al., 1996; Shepherd et al., 1997).

Perturbation of responsiveness to dermamyotome- and notochord-derived chemorepulsion in neuropilin-1-deficient DRG axons

Sema3A signals are mediated by direct binding to neuropilin-1 (He and Tessier-Lavigne, 1997; Kitsukawa et al., 1997; Kolodkin et al., 1997), and neuropilin-1 is expressed on DRG axons beginning at early stages of outgrowth in the chick and mouse embryo (Kawakami et al., 1996; Takagi et al., 1995) (Fig. 3H). Therefore, we have examined the role of neuropilin-1 in mediating dermamyotome- and notochord-derived chemorepulsion by culturing DRG explants derived from *neuropilin-1*-deficient (*neuropilin-1*−/−) mice. When E13.5 *neuropilin-1*+/+ or *neuropilin-1*+/− DRG explants were cultured with dermamyotome, notochord, or ventral spinal cord explants from E3 chick embryos (Fig. 3A–C), all three tissues strongly repelled these DRG axons. [the *p/d* values for the dermamyotome were 0.294 (+/+) and 0.332 (+/−), for the notochord were 0.234 (+/+) and 0.241 (+/−), and for the ventral spinal cord were 0.471 (+/+) and 0.500 (+/−)] (Fig. 3I–K). There were no differences in the *p/d* values between cocultures of *neuropilin-1*+/+and *neuropilin-1*+/− DRGs. These results suggest that both *neuropilin-1*+/+and *neuropilin-1*+/− DRGs respond to chemorepulsive activities derived from these explants. *Neuropilin-1*−/− DRG axons cultured with the dermamyotome and the notochord had increased *p/d* values of 0.971 and 0.832, respectively, showing a loss of responsiveness to chemorepellents derived from these explants (Fig. 3D, E, I, and J). By contrast, *neuropilin-1*−/− DRGs showed no significant reduction in chemorepulsive activities derived from the ventral spinal cord (the *p/d* value was 0.502, $P = 0.703$ compared to wild-type) (Fig. 3F and K). These results indicate that neuropilin-1 is involved in mediating notochord- and dermamyotome- but not ventral spinal cord-derived chemorepulsive activities at this stage.

Although *neuropilin-1*−/− DRG axons appear to lose responsiveness to both the dermamyotome- and the notochord-derived chemorepellents, long-term cultures revealed critical differences in the contribution of neuropilin-1 to the response of DRG axons. In 48-h cultures, *neuropilin-1*−/− DRG axons grew into and through the dermamyotome explants (see Fig. 3D). By contrast, although *neuropilin-1*−/− DRG axons grew toward the notochord, 19 of 20 samples did not actually reach it, leaving an axon-free zone around the notochord (Fig. 3G). Together, these studies suggest that the notochord exerts both Sema3A long-range chemorepulsive activity, via neuropilin-1, and an unidentified short-range chemorepulsive or contact-mediated inhibitory activity via other receptor(s), whereas the dermamyotome exerts chemorepulsive activities exclusively via neuropilin-1. We hypothesize that cell-surface molecules on DRG axons other than neuropilin-1 may mediate the ventral spinal cord-derived chemorepulsion at early stages.

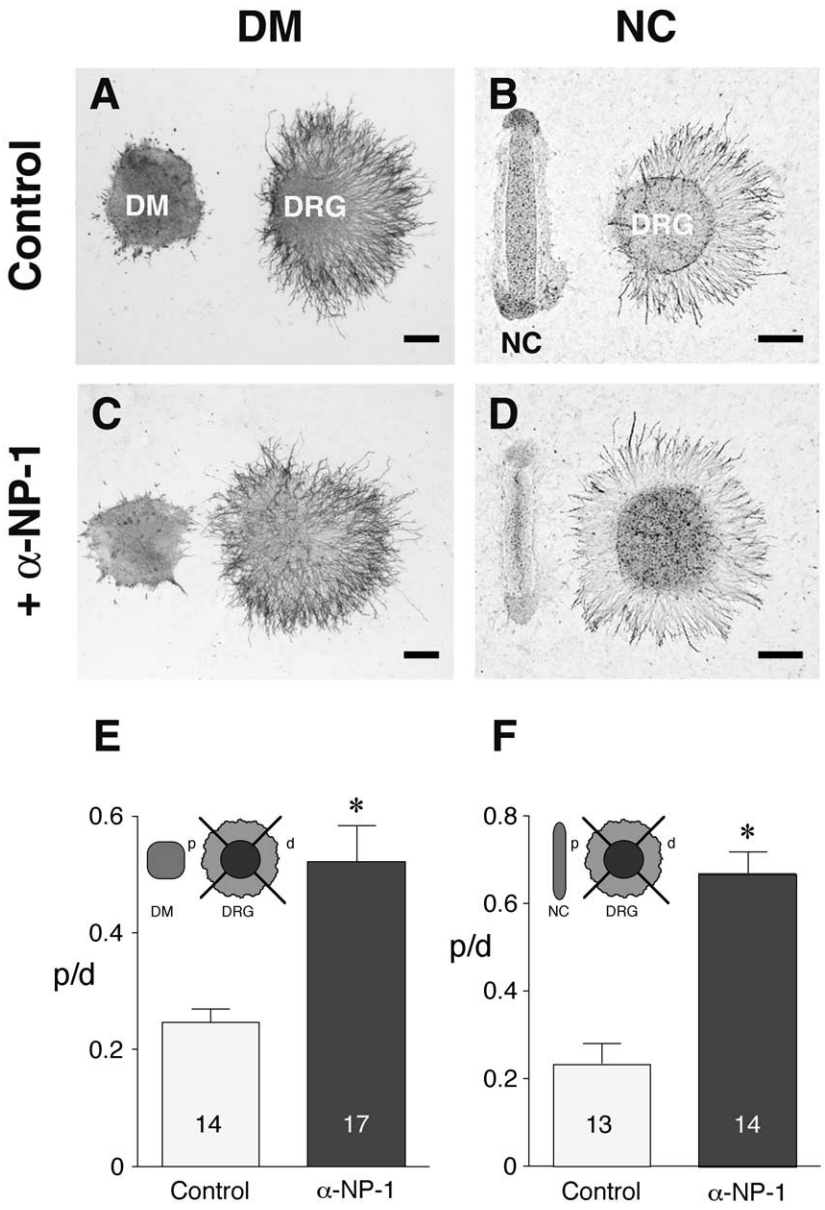

Fig. 4. The anti-neuropilin-1 antibody diminishes both dermamyotome- and notochord-derived chemorepulsion. (A–D) E5 chick DRG explants were cocultured with E3 chick dermamyotome (DM), or notochord (NC) explants in the absence (A, B) or presence (C, D) of the anti-neuropilin-1 antibody (α-NP-1). In the presence of anti-neuropilin-1, longer axons extended toward both the dermamyotome and the notochord. (E, F) Quantification of the effects of the anti-neuropilin-1 antibody on the dermamyotome-derived (E) or the notochord-derived (F) chemorepulsive activities for DRG axons. Addition of the anti-neuropilin-1 antibody significantly increased the *p/d* values in both two groups. The bars represent means + SEM and the number of cocultures is shown in the bar. $*P < 0.0001$, compared to the control group. Scale bars: 200 μm.

Anti-neuropilin-1 antibody diminishes the responses of DRG axons to both dermamyotome- and notochord-derived chemorepulsion

In the coculture experiments described above, we used mouse (or chick) DRG with surrounding tissues from either chick (or mouse) embryos. To control for the interspecies differences, we employed an anti-neuropilin-1 antibody which has been shown to block Sema3A-induced chemorepulsion and/or Sema3A-induced growth cone-collapsing activity in the rodent and the chick (He and Tessier-Lavigne, 1997; Vermeren et al., 2000). Cocultures of E5 chick DRGs and *Sema3A*-transfected COS cells showed that the antibody blocked Sema3A-induced chemorepulsion for chick DRG axons (data not shown). We cocultured E3 chick dermamyotome or notochord explants in combination with E5 chick DRG explants in either the presence or the absence of the anti-neuropilin-1 antibody. In the absence of the antibody, in agreement with our previous studies (Masuda et al., 2000; Nakamoto and Shiga, 1998), the dermamyo-

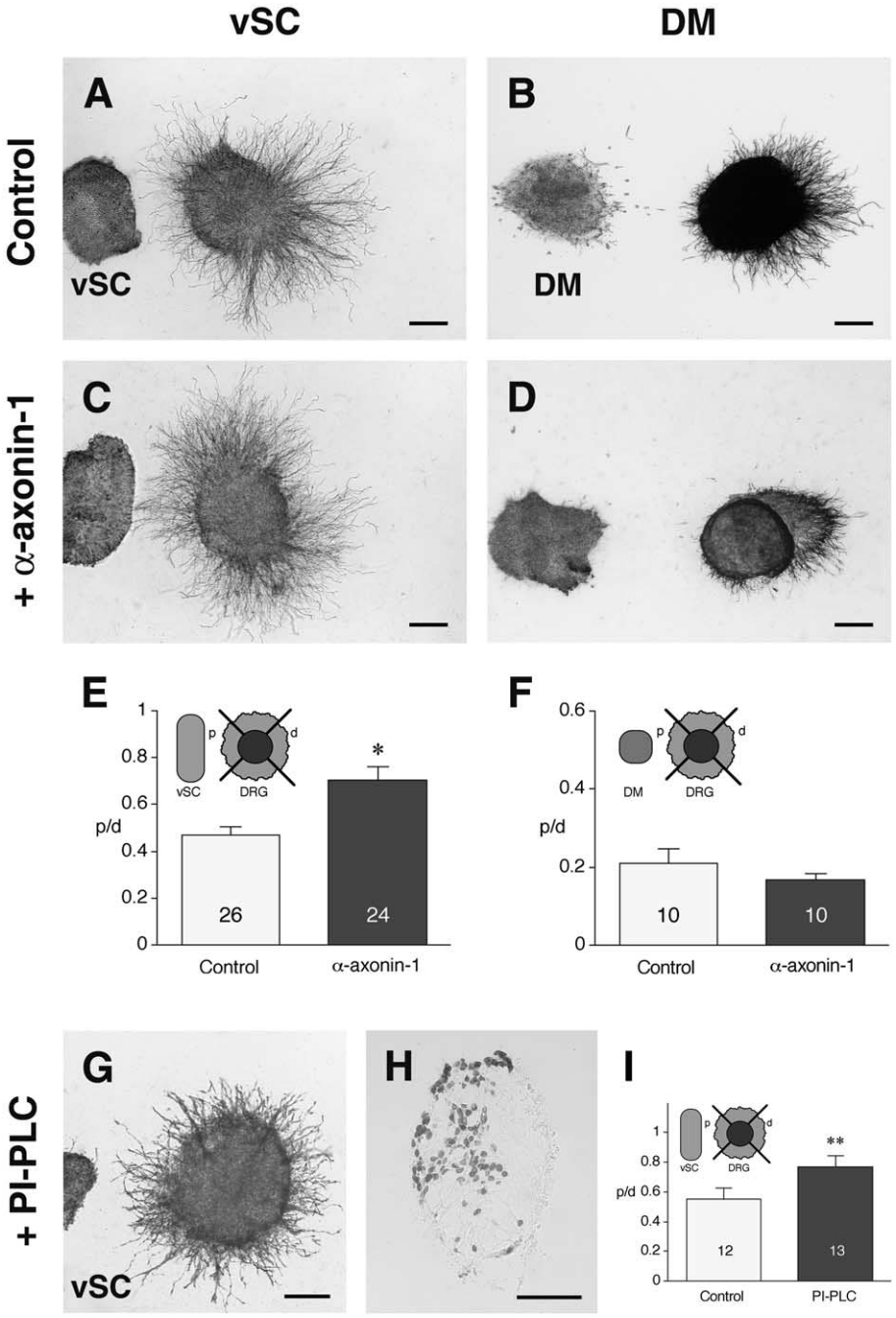

Fig. 5. Axonin-1/SC2 is required for ventral spinal cord-derived but not for dermamyotome-derived chemorepulsion. (A–D) E5 chick DRG explants were cocultured with E3 chick ventral spinal cord (vSC) or dermamyotome (DM) in the absence (A, B) or presence (C, D) of the anti-axonin-1/SC2 antibody (α-axonin-1). Adding the anti-axonin-1/SC2 antibody diminished ventral spinal cord-derived chemorepulsion for DRG axons, but not dermamyotome-derived chemorepulsion. (E, F) Quantification of the effect of the anti-axonin-1/SC2 antibody on chemorepulsion. Adding the anti-axonin-1/SC2 antibody significantly increased the *p/d* value in the cocultures of DRG explants with ventral spinal cord explants. (G, I) PI-PLC treatment significantly diminished the effect of ventral spinal cord-derived chemorepulsion to an extent similar to that of treatment with the anti-axonin-1/SC2 antibody. (H) PI-PLC treatment had no effect on the survival of the Islet-1-immunoreactive cells in the ventral spinal cord explants. The bars in E, F, and I represent means + SEM and the number of cocultures is shown in the bar. $*P < 0.001$, $**P < 0.05$, compared to the control group. Scale bars: 200 μm (A–D, and G), 50 μm (H).

tome and the notochord repelled DRG axons (Fig. 4A, B, E, and F). Addition of the anti-neuropilin-1 antibody into cocultures of both dermamyotome and notochord significantly increased the *p/d* values (Fig. 4C–F). The anti-neuropilin-1 antibody had no effect on the ventral spinal cord-derived chemorepulsive activities (data not shown). These findings are consistent with our results using DRGs from *neuropilin-1*−/− mice, indicating that neuropilin-1 on DRG axons is involved in mediating dermamyotome-and notochord-derived, but not ventral spinal cord-derived, chemorepulsion.

Anti-axonin-1/SC2 antibody diminishes the responses of DRG axons to ventral spinal cord-derived but not dermamyotome-derived chemorepulsion

We previously showed that axonin-1/SC2, which has been classified as a GPI-anchored cell adhesion molecule of the immunoglobulin superfamily (Zuellig et al., 1992), is expressed on DRG axons and mediates the notochord-derived chemorepulsive activities (Masuda et al., 2000). We thus investigated whether axonin-1/SC2 may also be required for mediating ventral spinal cord- or dermamyotome-derived chemorepulsion. We cocultured E5 chick DRG explants with E3 chick ventral spinal cord or dermamyotome explants in the presence or absence of an antibody against axonin-1/SC2. In the absence of the antibody, both the ventral spinal cord and the dermamyotome repelled DRG axons (Fig. 5A, B, E, and F). Addition of the anti-axonin-1/SC2 antibody to cocultures of the ventral spinal cord significantly increased the *p/d* value, but had no significant effect on cocultures with dermamyotome (Fig. 5C–F). The role of axonin-1/SC2 in mediating chemorepulsion was confirmed by PI-PLC treatment, which removes GPI-anchored molecules from the DRG axonal cell surface. PI-PLC treatment diminished the effect of ventral spinal cord-derived chemorepulsive activities to an extent similar to that of the treatment with the anti-axonin-1/SC2 antibody (Fig. 5G and I). To test whether the reduction of the ventral spinal cord-derived chemorepulsion is not due to the loss of viability of ventral spinal cord cells, we examined cells in the ventral spinal cord explants cultured for 24 h in the presence of PI-PLC. Immunohistochemical observations revealed the presence of Islet-1-immunoreactive cells which correspond to developing motor neurons in the ventral spinal cord explants (Ericson et al., 1992) (Fig. 5H). Therefore, it is unlikely that the ventral spinal cord cells were less viable by PI-PLC treatment. Together, these results further indicate that axonin-1/SC2 may be involved in mediating the chemorepulsive effect of the ventral spinal cord.

Perturbation of both neuropilin-1 and GPI-anchored cell surface molecules fails to completely inhibit the responsiveness of DRG axons to notochord-derived chemorepulsion

The present results together with the results from a previous study (Masuda et al., 2000) showed that both neuropilin-1 and axonin-1/SC2 participate in mediating notochord-derived chemorepulsion. Therefore, we examined whether perturbation of both the molecules might entirely block the responsiveness of DRG axons to chemorepulsion. As shown before in chick DRGs (Masuda et al., 2000), PI-PLC treatment of cocultures of E13.5 wild-type mouse DRGs and E3 chick notochords diminished the effect of chemorepulsive signal (Fig. 6A). However, even in cocultures of DRGs from E13.5 *neuropilin-1*−/− mice and E3 chick notochords in the presence of PI-PLC, DRG axons did not reach the notochord (21 of 25 samples left a distinct axon-free zone around the notochord) (Fig. 6B). These results strongly suggest the involvement of molecules other than neuropilin-1 and axonin-1/SC2 in mediating notochord-derived chemorepulsion.

To further investigate the role of axonin-1/SC2 in notochord-derived chemorepulsion, we examined whether axonin-1/SC2 might be involved in mediating the Sema3A-induced chemorepulsion. In a recent study it was reported that L1, another cell adhesion molecule in the immunoglobulin superfamily, participates in Sema3A signaling with neuropilin-1 (Castellani et al., 2000). *Sema3A*-transfected COS cell aggregates showed strong chemorepulsive activities for E5 chick DRG axons (Fig. 6C and F), whereas mock-transfected COS cell aggregates had no effect on DRG axons (Fig. 6E, see also Table 1). Addition of the anti-axonin-1/SC2 antibody to cocultures of DRGs and *Sema3A*-transfected COS cell aggregates had no significant effect on the *p/d* value (Fig. 6D and F). These results suggest that axonin-1/SC2 on DRG axons may not be required for mediating Sema3A signaling from the notochord. Because we do not know the epitopes that the antibody recognizes, further studies are needed to conclude the involvement of axonin-1/SC2 in Sema3A signaling.

Discussion

Our results clearly demonstrate that the initial trajectories of DRG axons are regulated by diverse chemorepulsive systems from surrounding tissues and that these are either dependent or independent of Sema3A/neuropilin-1 (Fig. 7). We also showed that the notochord exerts at least two different repulsive activities, a long-range diffusible activity and a short-range diffusible or contact-mediated one. Third, the presence of Sema3A-independent chemorepulsion in the early developing ventral spinal cord, together with data from previous studies (Messersmith et al., 1995; Shepherd et al., 1997), suggests there may be a transition from Sema3A-independent to -dependent chemorepulsion in the ventral spinal cord during development.

Chemorepulsion from the dermamyotome is exclusively dependent on Sema3A/neuropilin-1

Previous in vivo studies of *Sema3A* and *neuropilin-1* mutants demonstrated that DRG axons projected aberrantly from the lateral part of the DRG to the dermamyotome, suggesting that Sema3A/neuropilin-1 signaling plays an important role in dermamyotome-derived chemorepulsion (Kitsukawa et al., 1997; Taniguchi et al., 1997; White and Behar, 2000). However, these in vivo studies cannot exclude the involvement of other signals in dermamyotome-derived chemorepulsion. By using *Sema3A*- or *neuropilin-1*-deficient mice, we found that the radial outgrowth of DRG

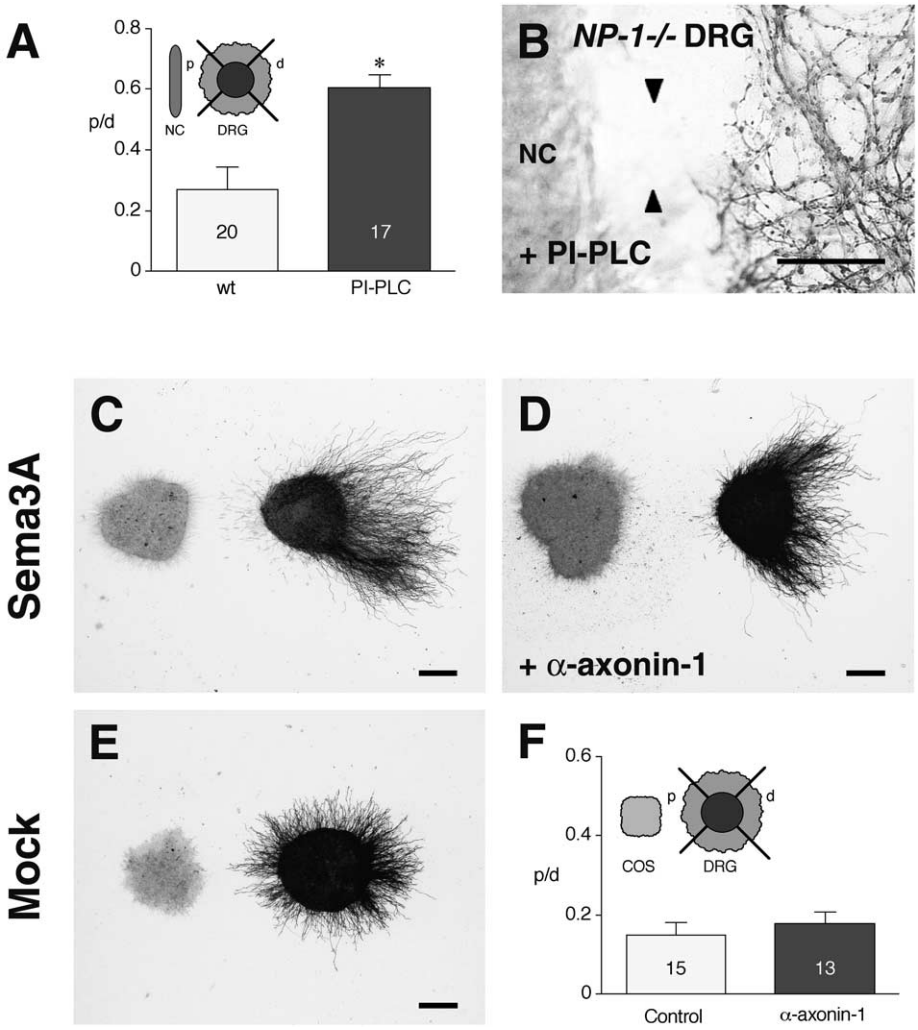

Fig. 6. Perturbation of both neuropilin-1 and GPI-anchored cell surface molecules fails to completely extinguish the responsiveness of DRG axons to notochord-derived chemorepulsion. (A) PI-PLC treatment of cocultures of E13.5 wild-type mouse DRG explants and E3 chick notochords significantly diminished notochord-derived chemorepulsion. (B) In the 48-h cocultures of E3 chick notochord (NC) and E13.5 *neuropilin-1 −/− (NP-1 −/−)* DRGs treated with PI-PLC, DRG axons did not reach the notochord and left an axon-free zone (arrowheads). (C, D) E5 chick DRG explants were cocultured with *Sema3A*- transfected COS cell aggregates (Sema3A) in the absence (C) or presence (D) of the anti-axonin-1/SC2 antibody (α-axonin-1). (E) Mock-transfected COS cell aggregates (Mock) had no effect on the outgrowth in the proximal quadrant of E5 DRG axons. (F) Quantification of the effect of the anti-axonin-1/SC2 antibody on Sema3A-induced chemorepulsion. Adding the anti-axonin-1/SC2 antibody had no effect on the *p/d* values in the cocultures of DRG explants with *Sema3A*- transfected COS cell aggregates. The bars in A and F represent means + SEM and the number of cocultures is shown in the bar. $*P < 0.001$, compared to the control group. Scale bars: 50 μm (B), 200 μm (C–E).

axons in cocultures with the dermamyotome provides the direct evidence that dermamyotome-derived chemorepulsion is entirely dependent on Sema3A via neuropilin-1.

It is interesting that the dermamyotome as well as the ventral spinal cord repels DRG axons at early developmental stages, but is subsequently innervated by them. There are several possible explanations for this developmental transition. First, the down-regulation of chemorepellents may permit later innervation. For example, the down-regulation of Sema3A in the dorsal spinal cord is thought to permit the innervation of the spinal gray matter by DRG axons (Fu et al., 2000; Püschel et al., 1996; Shepherd et al., 1997). Second, the down-regulation of receptors for chemorepellents may explain the transition (Pond et al., 2002; Shepherd et al., 1997). Finally, extrinsic modulation of chemorepulsion could be involved. Recently, several studies have shown that laminin-1, an extracellular matrix (ECM) molecule, can modulate the response of axons or growth cones to the diffusible axonal guidance molecule netrin-1 or Slit2 (Höpker et al., 1999; Nguyen-Ba-Charvet et al., 2001). Changes in ECM molecules surrounding the DRG may modulate intracellular cyclic nucleotide levels in DRG growth cones, resulting in an alteration of the DRG axonal response to chemorepellents.

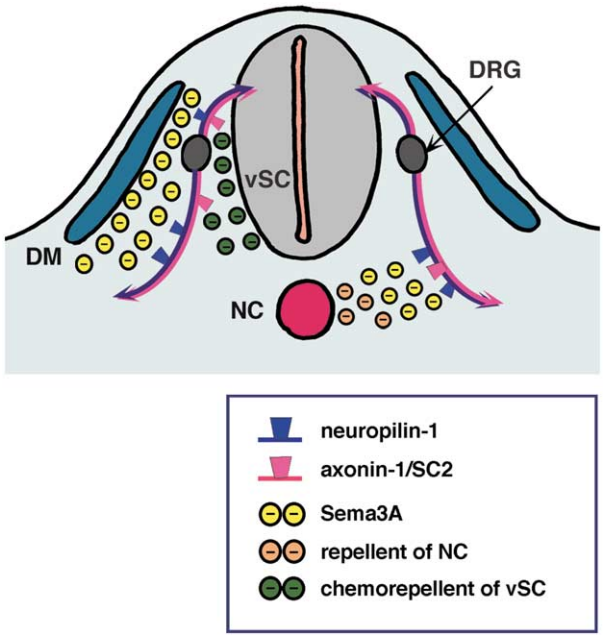

Fig. 7. A schematic diagram summarizing the molecular nature of the repulsion from the dermamyotome, the ventral spinal cord (left side), and the notochord (right side). Neuropilin-1 (blue) and axonin-1/SC2 (red) are expressed on DRG axons. Both the dermamyotome (DM) and the notochord (NC) exert Sema3A-induced chemorepulsion (yellow circles) on DRG axons via neuropilin-1. The notochord exerts an unknown repulsion other than Sema3A (orange circles) on DRG axons via axonin-1/SC2. The ventral spinal cord (vSC) exerts an unknown chemorepulsion (green circles) via axonin-1/SC2, but independent of Sema3A/neuropilin-1.

The notochord exerts multiple repulsive activities

Previous in vivo studies of *Sema3A*- or *neuropilin-1*-deficient mice found normal projections of DRG axons in relation to the notochord, suggesting that Sema3A/neuropilin-1 signaling may not be involved in notochord-derived chemorepulsive activity (Kitsukawa et al., 1997; Taniguchi et al., 1997; White and Behar, 2000). By contrast, we provide strong evidence indicating that Sema3A/neuropilin-1 plays a major role in notochord-derived chemorepulsion. The discrepancy between the in vivo analyses of mutant mice and our in vitro coculture study may be explained by the repulsive activities of sclerotomal cells around the notochord. It has been shown that these sclerotomal cells (perinotochordal mesenchymal cells) act as a barrier that inhibits the ingrowth of DRG axons and spinal motor axons and that this effect may be mediated by chondroitin sulfate proteoglycans (CSPGs) (Oakley and Tosney, 1991; Tosney and Oakley, 1990).

We observe that the *Sema3A*-deficient notochord retains weak repulsive activities, and that *neuropilin-1*-deficient DRG axons grow toward the notochord, but leave an axon-free zone around it. These results suggest that the notochord exerts both a long-range/diffusible chemorepulsion which is dependent on Sema3A/neuropilin-1 signaling and a short-range chemorepulsion or contact-mediated repulsion which is independent of Sema3A/neuropilin-1. CSPGs are a promising candidate for the notochord-derived short-range chemo- or contact-mediated repellent. CSPGs are potent inhibitors of DRG axonal growth (Dou and Levine, 1995; Fichard et al., 1991; Katoh-Semba et al., 1995; Snow and Letourneau, 1992), and the notochord secretes CSPGs (Bundy et al., 1998; Domowicz et al., 1995; Perissinotto et al., 2000). However, Keynes et al., (1997) failed to diminish the notochord-derived chemorepulsion by the treatment of cocultures with a neutralizing anti-CSPG antibody or with the glycosylation inhibitor β-D-xyloside. It is possible that the contribution of CSPGs in notochord-derived chemorepulsion may be masked by the strong chemorepulsive activity of Sema3A.

We have previously shown that axonin-1/SC2, a GPI-anchored cell adhesion molecule, is involved in mediating notochord-derived chemorepulsion (Masuda et al., 2000). In the present study, we demonstrated that axonin-1/SC2 may not be involved in mediating Sema3A signaling. Furthermore, we failed to completely abolish notochord-derived repulsion by perturbing both neuropilin-1 and GPI-anchored cell-surface molecules. Taken together, these results suggest that the notochord repels DRG axons mainly by Sema3A via neuropilin-1, but that other repulsive signals that do not require either Sema3A/neuropilin-1 or axonin-1/SC2 are also involved.

Ventral spinal cord-derived chemorepulsion is developmentally regulated

Our previous results showed that E3 chick ventral spinal cord does not repel E9 DRG axons which respond to Sema3A (Nakamoto and Shiga, 1998), indicating that Sema3A may not be responsible for ventral spinal cord-derived chemorepulsion during the initial stage of DRG axonal growth. The in situ hybridization analyses revealed that *Sema3A* mRNA expression does not occur in the chick spinal cord before E4 (Shepherd et al., 1996 and the present study). In addition, the *Sema3A*-deficient ventral spinal cord retains chemorepulsive activity for DRG axons, and *neuropilin-1*-deficient DRG axons responded to chemorepellents from the ventral spinal cord. These data provide strong evidence that ventral spinal cord-derived chemorepulsion in the *initial* stage of DRG axonal growth is independent of Sema3A/neuropilin-1. Sema3A/neuropilin-1 plays important roles in the central projection of DRG axons within the spinal cord. Thus, in vitro studies have revealed that Sema3A is involved in the regulation of the timing of DRG axonal invasion into the dorsal horn of the spinal cord (Fu et al., 2000; Püschel et al., 1996; Shepherd et al., 1997) and in the lamina-specific projection of NGF-dependent DRG axons within the spinal gray matter (Messersmith et al., 1995). Our results suggest that the ventral spinal cord secretes several different chemorepellents for DRG axons and that a transition occurs in ventral spinal cord-derived che-

morepulsion from one independent of Sema3A/neuropilin-1 to one dependent on such signaling.

We have also found that axonin-1/SC2 may mediate ventral spinal cord- but not dermamyotome-derived chemorepulsion. The role of axonin-1/SC2 in chemorepulsion from these tissues remains to be elucidated. Putative chemorepellents mediated by axonin-1/SC2 have not been reported. Axonin-1/SC2 may not be involved in Sema3A-induced chemorepulsion and although some CSPGs have been shown to bind axonin-1/SC2 (Milev et al., 1996), it is unlikely that axonin-1/SC2 is involved in CSPGs-induced repulsive activities. The anti-axonin-1/SC2 antibody used here diminished the responsiveness of DRG axons to ventral spinal cord-derived chemorepulsion, whereas inhibition of CSPGs by chondroitinase ABC treatment had no effect on ventral spinal cord-derived chemorepulsion (Masuda and Shiga, unpublished observations).

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